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BEX1/ARF1A1C is Required for BFA-Sensitive Recycling of PIN Auxin Transporters and Auxin-Mediated Development in Arabidopsis

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Correct positioning of membrane proteins is an essential process in eukaryotic organisms. The plant hormone auxin is distributed through intercellular transport and triggers various cellular responses. Auxin transporters of the PIN-FORMED (PIN) family localize asymmetrically at the plasma membrane (PM) and mediate the directional transport of auxin between cells. A fungal toxin, brefeldin A (BFA), inhibits a subset of guanine nucleotide exchange factors for ADP-ribosylation factor small GTPases (ARF GEFs) including GNOM, which plays a major role in localization of PIN1 predominantly to the basal side of the PM. The Arabidopsis genome encodes 19 ARF-related putative GTPases. However, ARF components involved in PIN1 localization have been genetically poorly defined. Using a fluorescence imaging-based forward genetic approach, we identified an Arabidopsis mutant, *bfa-visualized exocytic trafficking defective1* (*bex1*), in which PM localization of PIN1–green fluorescent protein (GFP) as well as development is hypersensitive to BFA. We found that in *bex1* a member of the *ARF1* gene family, *ARF1A1C*, was mutated. ARF1A1C localizes to the *trans*-Golgi network/early endosome and Golgi apparatus, acts synergistically to BEN1/MIN7 ARF GEF and is important for PIN recycling to the PM. Consistent with the developmental importance of PIN proteins, functional interference with ARF1 resulted in an impaired auxin response gradient and various developmental defects including embryonic patterning defects and growth arrest. Our results show that ARF1A1C is essential for recycling of PIN auxin transporters and for various auxin-dependent developmental processes.

Keywords: *Arabidopsis thaliana* • Auxin • Embryogenesis • Exocytosis • PIN-FORMED.

Abbreviations: ARF, ADP ribosylation factor; *bex1*, *bfa-visualized exocytic trafficking defective 1*; BFA, brefeldin A; EE, early endosome; ER, endoplasmic reticulum; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; NPA, *N*-1-naphthylphthalamic acid; PIN, PIN-FORMED; PM, plasma membrane; QC, quiescent center; TGN, *trans*-Golgi network; YFP, yellow fluorescent protein.

Introduction

Local accumulation or depletion of the plant hormone auxin is critical in regulating multiple developmental processes in plants (Mockaitis and Estelle 2008, Vanneste and Friml 2009). An auxin gradient is formed by the combined actions of local auxin biosynthesis and intercellular auxin transport (Vanneste and Friml 2009). Genetic studies and subsequent functional studies have identified influx and efflux transporters for auxin (Petrášek and Friml 2009). Remarkably, the PIN-FORMED (PIN) family of auxin efflux transporters localize asymmetrically in the plasma membrane (PM) of cells belonging to different tissues in a manner consistent with the known direction of auxin flow (Tanaka et al. 2006, Vanneste and Friml 2009). Moreover, a strong casual link between auxin distribution and asymmetric PIN localization has been demonstrated by experiments where the amino acid sequence and phosphorylation status of the transporters were manipulated, affecting their PM polarity (Friml et al. 2004, Wiśniewska et al. 2006, Michniewicz et al. 2007, Huang et al. 2010, Zhang et al. 2010). Thus, regulation of polar PIN localization, their amount at the PM and potentially their activities are critical for various auxin-dependent processes.

It has been demonstrated that localization of PIN1 protein in root vascular tissues mainly involves an intracellular trafficking

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pathway, which is sensitive to the fungal toxin brefeldin A (BFA) (Geldner et al. 2001). In terms of PIN1 trafficking, the major target of BFA is the BFA-sensitive guanine nucleotide exchange factor for ADP-ribosylation factor small GTPases (ARF GEF) GNOM (Geldner et al. 2003). In addition, exocytosis of PIN1 involves interactor of constitutive active ROP/ROP interactive partner1 (ICR1/RIP1) (Hazak et al. 2010), exocyst components (Lavy et al. 2007, Drdova et al. 2013) and RabA1b (Feraru et al. 2012). However, the molecular components involved in the PM distribution of PIN1 protein are not fully understood. In order to identify additional molecular components involved in the PM distribution of PIN1 protein, we performed a fluorescence imaging-based screen for Arabidopsis mutants that overaccumulate PIN1–green fluorescent protein (GFP) proteins in intracellular compartments in the presence of BFA. From this mutant screen, we identified a BFA-hypersensitive mutant *bfa-visualized exocytic trafficking defective 1* (*bex1*). Molecular cloning of the *bex1* gene revealed that a dominant mutation in a gene encoding a small GTP-binding protein of the ARF family, *ARF1A1C*, was responsible for the mutant phenotypes. Based on our phenotypic characterization of *bex1* mutant and transgenic plants expressing mutated ARF1A1C, we propose that ARF1A1C is critical for regulating intracellular trafficking, including targeting of PIN1 proteins to the PM.

Results

Screening for mutants with altered PIN1–GFP trafficking

When PIN1–GFP-expressing wild-type plants were treated for a short time (50 μM, 1.5 h) with BFA, basally (rootward) localized PIN1–GFP accumulated in intracellular compartments (BFA bodies; **Fig. 1A**, upper panel, **Fig. 1B**). Longer treatment with BFA is known to cause relocation of PIN1 to the apical (shootward) PM (Kleine-Vehn et al. 2008a). Consistent with this, PIN1–GFP after long-term BFA treatment (50 μM, 12 h) was mainly detected at the PM in wild-type root vascular cells (**Fig. 1A**, upper panel, **Fig. 1B**). We reasoned that this treatment allows us to perform a directed screen for exocytosis-related components of the subcellular machinery. By screening approximately 39,200 ethylmethane sulfonate (EMS)-mutagenized M_2 seedlings, we have identified a mutant in which clear agglomerations of PIN1–GFP signals were detected after the long-term BFA treatment, and named it *bfa-visualized exocytosis defective1* (*bex1*; **Fig. 1A, B**). Genetic crosses between *bex1* mutant and wild-type plants as well as subsequent characterization of F_1 seedlings revealed that 32 out of 33 F_1 seedlings showed the *bex1* mutant phenotype, indicating that *bex1* is a dominant mutant.

The *bex1* mutant is defective in intracellular trafficking of PIN proteins

Treatment with a lower concentration of BFA (10 μM, 1 and 3 h) did not visibly diminish the PM localization of PIN1–GFP in the wild type (**Fig. 1C**, upper panels). In contrast, under the same conditions, the *bex1* mutant accumulated PIN1–GFP in intracellular compartments and the PM signal was clearly reduced (10 μM, 3 h: **Fig. 1C**, lower panels, **Fig. 1D**). This observation implies that intracellular trafficking from endosomes to the PM was affected by the *bex1* mutation. To gain further insight into the intracellular trafficking defect, we performed a BFA washout experiment (**Supplementary Fig. S1A**). In wild-type root vascular cells, PIN1–GFP accumulated in BFA compartments after treatment with BFA (50 μM, 1.5 h), but the intracellular aggregates rapidly decreased and the PM signals were recovered when BFA was washed away (**Supplementary Fig. S1A**, upper panels). In contrast, in the *bex1* mutant background, the recovery of PM signals was less clear after BFA washout (**Supplementary Fig. S1A**, lower panels). These results suggest that the *bex1* mutation affects transport of PIN1–GFP, including exocytic trafficking, at the level of BFA-sensitive compartments.

Next, we tested if the localization of PIN2 is also affected by *bex1* mutation. Treatment with a low concentration of BFA (10 μM, 3 h) caused a moderate intracellular accumulation of PIN2 in wild-type root epidermal cells. In *bex1* mutant cells, however, intracellular accumulation of PIN2 was more pronounced than in wild-type cells (**Fig. 2D**). Consistent with this observation, when introduced in the *bex1* mutant background, PIN2–GFP persisted more in the BFA compartment after BFA washout, in comparison with PIN2–GFP in control cells (**Supplementary Fig. S1B**). As it is known that BFA at high concentrations inhibits vacuolar targeting of PIN2 in the wild-type background (Kleine-Vehn et al. 2008b), we tested whether this process is hypersensitive in the *bex1* mutant background. As shown in **Supplementary Fig. S2**, PIN2–GFP in the control line (*PIN2-GFP; eir1*) exhibited intracellular signals typical for vacuolar cargo proteins that can be visualized by keeping seedlings in darkness (Tamura et al. 2003). Consistent with previous observations (Kleine-Vehn et al. 2008b), a low concentration of BFA did not visibly interfere with vacuolar targeting of PIN2–GFP in the control line. In contrast, a low concentration of BFA dramatically reduced the vacuolar localization of GFP signals in the *bex1* mutant background, suggesting that not only recycling to the PM but also vacuolar targeting of PIN2–GFP was hypersensitive to BFA (**Supplementary Fig. S2**).

Molecular cloning reveals that *bex1* is a dominant allele of *ARF1A1C*

In order to identify a mutation responsible for *bex1* phenotypes, we performed positional cloning based on the BFA-hypersensitive phenotype of PIN1–GFP intracellular accumulation. Utilizing a PCR-based single nucleotide polymorphism genotyping approach, we were able to identify a chromosomal location genetically linked to the BFA-hypersensitive phenotype of the *bex1* mutant. As a result, we found a 42 kb region on chromosome 2 which always co-segregated with the *bex1* locus

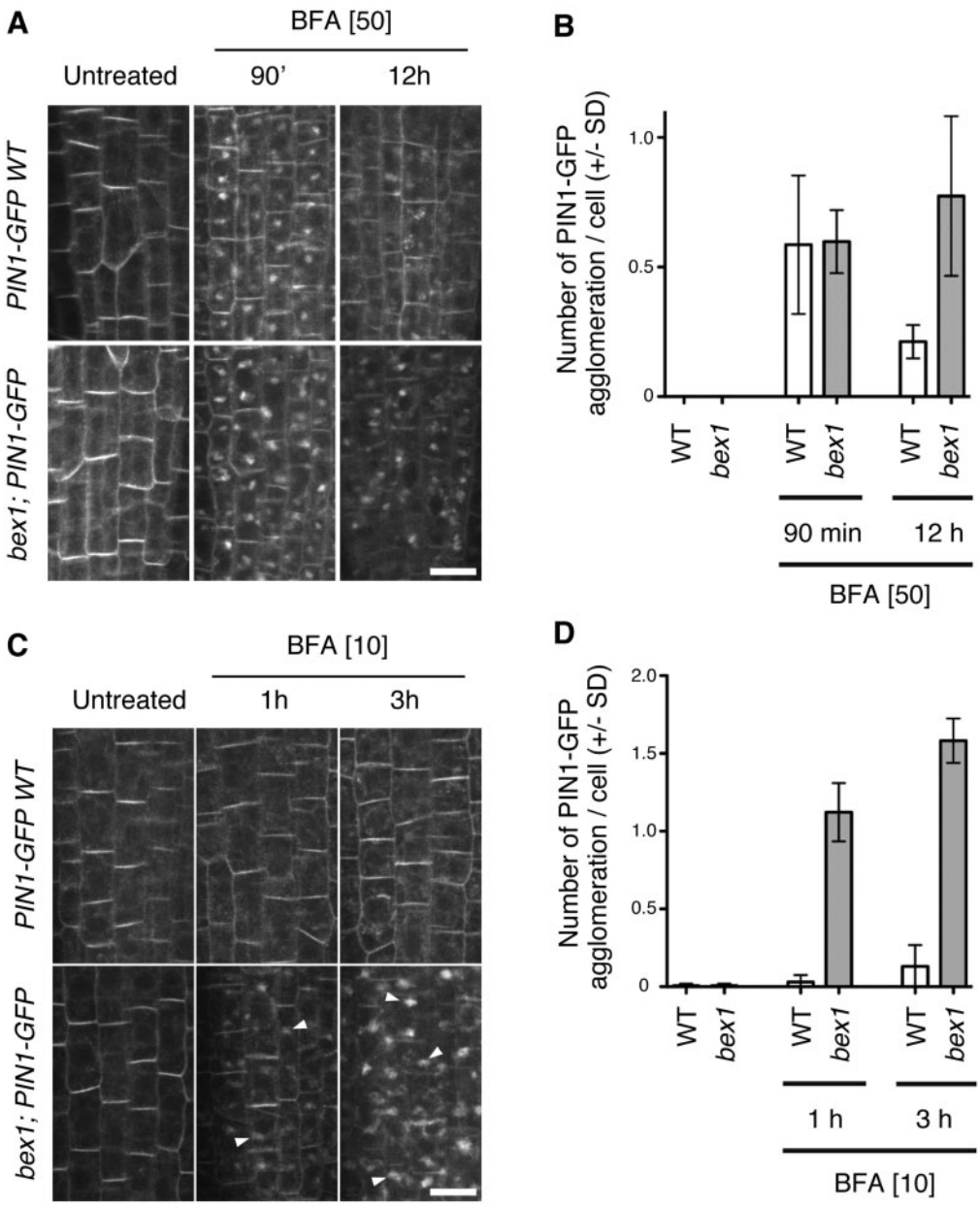

Fig. 1 The *bex1* mutant exhibits altered responses to BFA. (A) Localization of PIN1–GFP in untreated seedlings (left-hand panels) and seedlings treated with BFA (50 μM) in liquid media (middle and right-hand panels). Intracellular accumulations of PIN1–GFP in root vascular cells are more pronounced after 12 h BFA treatment in the *bex1* mutant (lower right panel). (B) Quantification of intracellular PIN1–GFP accumulation after a short treatment (90 min) and longer treatment (12 h) with BFA (50 μM). (C) Effect of a lower concentration of BFA (10 μM) on PIN1–GFP localization. Whereas PIN1–GFP signals were mainly detected at the PM in wild-type root vascular cells (upper panels), the auxin efflux carrier accumulated in intracellular compartments (arrowheads) while its PM signals were reduced in *bex1* (lower panels). (D) The frequency of PIN1–GFP agglomeration after treatment with a low concentration of BFA (10 μM). Scale bars = 10 μm.

(**Fig. 2A**). Sequencing of genes in this interval revealed that the open reading frame At2g47170 encoding ARF1A1C had a point mutation, causing an amino acid substitution (L34F) in the gene product (**Fig. 2B, C**). Amino acid sequence alignment of ARF1A1C with small G-proteins from yeast revealed that the mutated leucine residue is well conserved in different small G-proteins (**Fig. 2C**), highlighting the potential importance of this region. Indeed, the site of *bex1* mutation is located within a domain called switch 1, which is involved in a structural change of ARF1 upon GDP and GTP exchange (Gillingham and Munro 2007). Because the Arabidopsis genome encodes six members belonging to the ARF1A1 subclass, which are highly conserved in terms of amino acid sequence (98–99% identical to the ARF1A1C sequence), the *bex1* mutation might have resulted in a dominant inhibitory mutation causing a relatively strong trafficking defect.

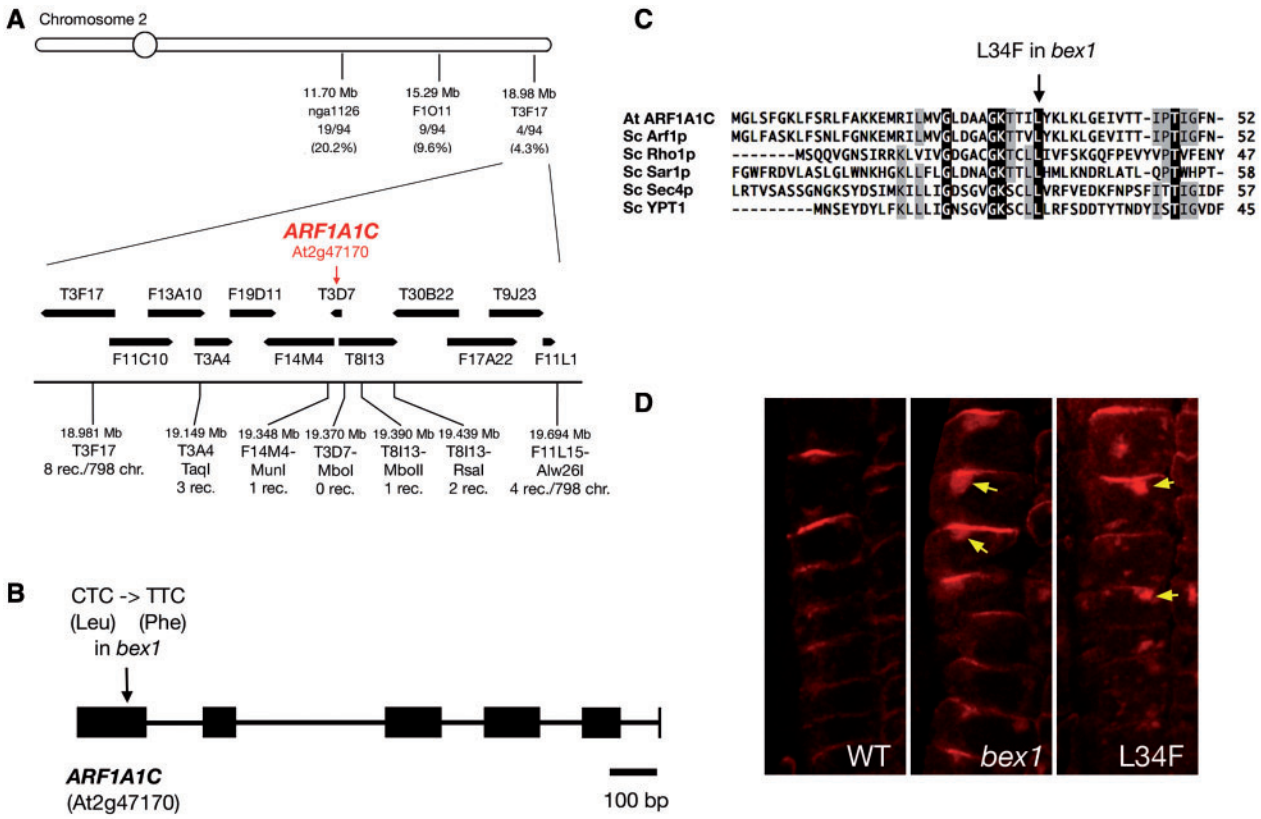

Fig. 2 Molecular cloning reveals that *bex1* contains a mutation in the *ARF1A1C* gene. (A) Map position of the *bex1* mutation defined by map-based cloning. Relative positions of BAC (bacterial artificial chromosome) contigs on chromosome 2 are shown. The numbers of recombinations between the *bex1* locus and markers are indicated. (B) Gene structure of *ARF1A1C* with the position of nucleotide substitution. (C) Alignment of partial amino acid sequences of Arabidopsis ARF1A1C and small GTPases from yeast. (D) PIN2 immunostaining of wild-type, *bex1* and transgenic plants expressing the ARF1A1C^{L34F} construct, treated with 10 μM BFA for 3 h. Arrows indicate strongly agglomerated PIN2 signals.

To test this possibility, we created an *ARF1A1C* construct with *bex1* mutation (*ARF1A1C*^{L34F}) and introduced it into wild-type Arabidopsis plants under the control of the *ARF1A1C* promoter. This construct recapitulated *bex1* phenotypes in terms of PIN2 localization (**Fig. 2D**), indicating that the ARF1A1C L34F mutation is responsible for the BFA hypersensitivity. ARF1 is an evolutionarily conserved small GTPase protein, which regulates intracellular trafficking at the Golgi apparatus and endosomal compartments (Gillingham and Munro 2007). Currently available circumstantial evidence supports the roles of ARF1 in multiple trafficking processes (Lee et al. 2002, Takeuchi et al. 2002, Pimpl et al. 2003, Xu and Scheres 2005, Kleine-Vehn et al. 2008a, Kleine-Vehn et al. 2008b) and their potential developmental impact in plants (Geldner et al. 2001, Geldner et al. 2003, Gebbie et al. 2005, Xu and Scheres 2005, Richter et al. 2007, Teh and Moore 2007, Tanaka et al. 2009). Our result is thus consistent with previously published data and indicates a clear biological relevance of ARF1A1C function in PIN protein trafficking.

ARF1A1C co-localizes with multiple ARF GEFs

In Arabidopsis root epidermal cells, ARF1A1C–GFP co-localizes with *trans*-Golgi network (TGN)/early endosome (EE) and Golgi apparatus markers (Xu and Scheres 2005, Tanaka et al. 2009). To examine the subcellular localization of ARF1A1C in more detail, we performed immunolabeling experiments using anti-ARF1A1C antibody on Arabidopsis root epidermal cells expressing ARF GEFs fused to fluorescent reporters. The control immunostaining with this antibody resulted in punctate staining, which overlapped well with ARF1A1C–GFP signals (**Fig. 3A, B**). Fluorescent protein-fused ARF GEFs [i.e. BEN1–GFP, GNOM–GFP and GNL1–yellow fluorescent protein (YFP)] overlapped with anti-ARF1 antibody staining (**Fig. 3A, B**). Partial co-localization of ARF1 with GNOM–GFP suggests that some population of ARF1A1C localizes to GNOM–GFP-labeled compartments, which might represent recycling endosomes. In addition, as BEN1 and GNL1 mainly localize to the TGN/EE (Tanaka et al. 2009) and Golgi apparatus (Richter et al. 2007, Teh and Moore 2007), respectively, these results suggest that ARF1A1C localizes to intracellular compartments including the TGN/EE and Golgi apparatus, which are labeled with these ARF GEFs.

Consistently, immunostaining experiments confirmed that ARF1A1C–GFP co-localizes with MIN7/BEN1 and a Golgi marker SEC21/γ-COP (**Fig. 4A**; **Supplementary Fig. S3**). Notably, ARF1A1C did not co-localize with anti-BiP staining,

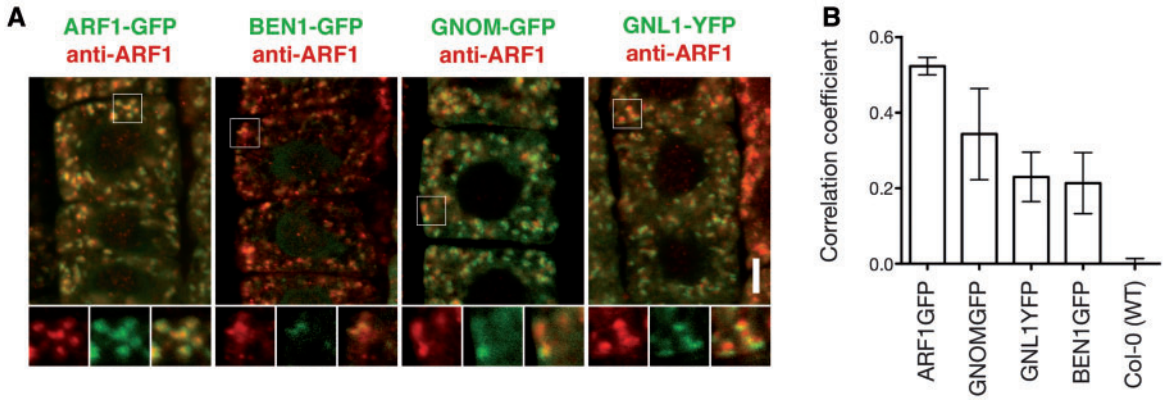

Fig. 3 ARF1A1C partially co-localizes with multiple ARF GEFs. (A) Double labeling of root epidermal cells expressing ARF GEFs tagged with fluorescent reporters and anti-ARF1A1C immunostaining. Fluorescence signals from XFPs were directly detected (green). Anti-ARF1A1C signals are shown in red. As a control, ARF1A1C–GFP was stained with anti-ARF1 antibody, revealing a major overlap between GFP signals and antibody staining. Scale bar = 5 μm. (B) Quantification of co-localization between anti-ARF1 antibody staining and XFP-fused ARF1 and ARF GEFs. Average values and the standard deviation of the correlation coefficient are indicated.

which labels the endoplasmic reticulum (ER) (**Supplementary Fig. S3**). When moderately expressed, ARF1^{L34F}–GFP also co-localized with MIN7/BEN1 and SEC21/γ-COP, but not with BiP, suggesting that L34F mutation did not abolish its native subcellular localization (**Fig. 4A**; **Supplementary Fig. S3**, middle panels). However, in the seedling roots with strong ARF1^{L34F}–GFP florescence, GFP signals were often observed in agglomerated endosomes, which were labeled with MIN7/BEN1 and SEC21/γ-COP (**Fig. 4A**; **Supplementary Fig. S3**). Consistently, immunoblotting experiments using anti-ARF1A1C antibody confirmed that ARF1^{L34F}–GFP proteins were highly expressed in the transgenic lines, which showed relatively strong BFA-hypersensitive phenotypes (lines 1, 7 and 8: **Fig. 4B**). In such ARF1^{L34F}-overexpressing lines, albeit at low frequency (16% in line 1, $n = 147$; 12% in line 8, $n = 57$), seedling growth was severely retarded (**Fig. 4C**). Taken together, these results suggest that L34F mutation did not primarily affect the subcellular localization of ARF1 at the TGN/EE and Golgi apparatus but overexpression of the mutated protein interfered with ARF1-regulated intracellular trafficking and caused severe developmental defects.

Genetic interaction between *bex1* and *ben1*

Based on these results, we speculated that there was a possible role for ARF1A1C to function at multiple compartments together with several different ARF GEFs regulating intracellular trafficking processes. As shown in **Fig. 5A**, the *bex1* mutant overaccumulates PIN1–GFP in the BFA compartment upon treatment with BFA (10 μM, 3 h), whereas intracellular agglomeration of PIN1–GFP was not discernible in wild-type and *ben1* seedling roots under the same conditions. BEN1 ARF GEF is involved in trafficking at the TGN/EE and required for PIN1–GFP accumulation in the BFA compartment (Tanaka et al. 2009). If the *bex1* mutation affects endocytic recycling of PIN1 at the same level as the site of BEN1 action and/or at a later step, we expect that the *ben1* mutation is epistatic to *bex1* in terms of endocytic recycling of PIN1 protein. Indeed, following BFA treatment, intracellular agglomeration in *ben1;bex1* double mutant cells was less pronounced as compared with the *bex1* mutant (**Fig. 5A**), suggesting that BEN1 ARF GEF and BEX1/ARF1A1C function in an interconnected trafficking pathway.

Given the localization of ARF1A1C in the early endosomal pathway and potential redundancy within the ARF1A1 subclass, we speculated that the phenotypes of the *bex1* mutant might be enhanced by inactivation of the early endosomal ARF GEF, BEN1 (Tanaka et al. 2009). Intriguingly, *bex1* and *ben1* single mutant plants had only mild growth defects whereas *bex1; ben1* double homozygous plants were extremely small at maturity (**Fig. 5B**), indicating the developmental importance of these two components.

ARF1A1C is involved in auxin-related developmental processes

The BFA-hypersensitive PIN trafficking defect of *bex1* prompted us to examine whether the mutant shows any auxin-related developmental defects. For this purpose, we grew *bex1* plants on vertical plates with solid media containing various concentrations of BFA. Wild-type seedlings grown at moderate BFA concentration (e.g. 5 μM) showed only slight root growth inhibition, whereas *bex1* mutant seedlings stopped growing under the same conditions (**Fig. 6A, B**). Expression of ARF1A1C^{L34F} in the wild-type background resulted in a similar growth defect on BFA-containing media, confirming the dominant effect of *ARF1A1C/BEX1* L34F mutation on shoot and root development (**Fig. 6C**). In the wild-type root tip, the *DR5rev::GFP* auxin response reporter (Friml et al. 2003b) was mainly detected in the quiescent center (QC) and columella root cap cells, and BFA treatment (25 μM, 20 h) only slightly reduced the GFP signals (**Fig. 6D**). In contrast, in BFA-treated

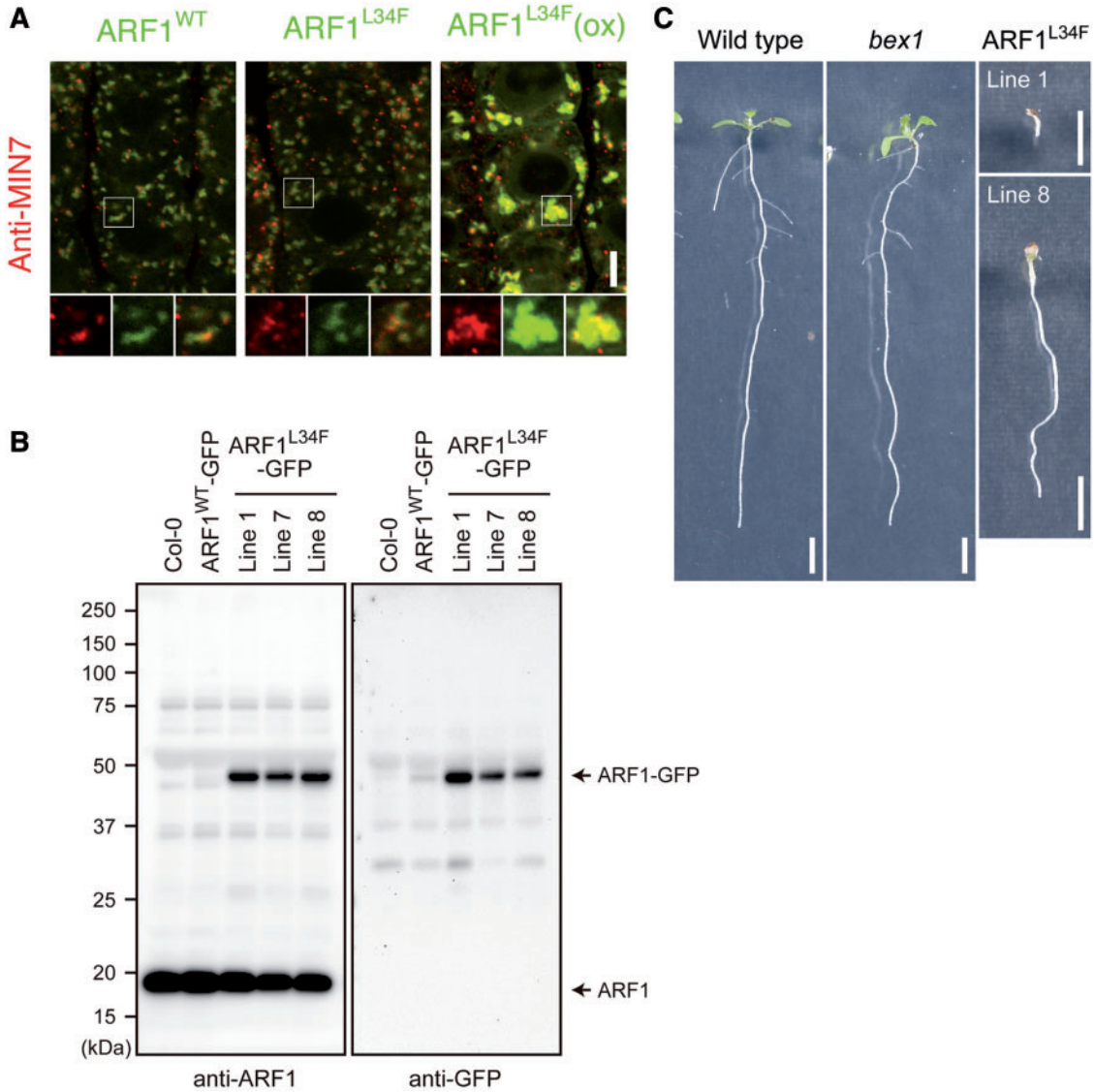

Fig. 4 Overexpression of ARF1A1C with the L34F mutation causes agglomeration of intracellular compartments. (A) Localization of the wild-type ARF1A1C–GFP and ARF1A1C^{L34F}–GFP (green) with anti-MIN7/BEN1 antibody staining (red). ARF1^{L34F}–GFP localization patterns in moderately expressing seedling roots (line 7) were similar to those of ARF1^{WT}–GFP, whereas in the seedlings overexpressing ARF1^{L34F}–GFP (line 1), GFP signals were agglomerated (right panel). Lines 7 and 8 also segregated seedlings with strong and agglomerated GFP signals. Scale bar = 5 μm. (B) Immunoblotting experiments confirm that ARF1^{L34F}–GFP of the expected size was expressed in transgenic lines (lines 1, 7 and 8). Anti-ARF1A1C antibody mainly detected endogenous protein at around 20 kDa and ARF1–GFP at around 48 kDa (left panel). Anti-GFP antibody mainly detected ARF1A1C–GFP fusion proteins, which were specific to transgenic plants (right panel). (C) Morphology of vertically grown plantlets (10 d old). Severely affected *ARF1^{L34F}-GFP*-expressing plantlets exhibited arrested or retarded growth phenotypes. Scale bars = 5 mm.

bex1 roots, *DR5rev::GFP* was more broadly expressed, indicating that the auxin response gradient was impaired (**Fig. 6D**). Notably, *bex1* seedlings showed normal sensitivity to the auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA; **Fig. 6A**). Thus it seems that *bex1* mutants are specifically compromised in intracellular trafficking processes.

A point mutation in the domain responsible for GDP/GTP exchange, critical for regulating ARF1 function, renders the protein locked in a GDP- or GTP-bound state (Xu and Scheres 2005, Gillingham and Munro 2007). Expression of ARF1^{T31N}, a GDP-locked mutant, has a dominant negative effect, resulting in a more dispersed localization pattern of the COPI component in plant cells (Stefano et al. 2006) (**Supplementary Fig. S4A**). Also, ARF1^{Q71L}, a GTP-locked mutant, inhibits root growth and root hair formation when expressed in Arabidopsis roots (Xu and Scheres 2005). We tried to uncover the additional functions of ARF1, in terms of PIN trafficking and development, by overexpressing the mutated ARF1A1C. When the GTP-locked ARF1A1C^{Q71L} mutant fused to fluorescent protein was expressed under a heat shock promoter, PIN1 protein

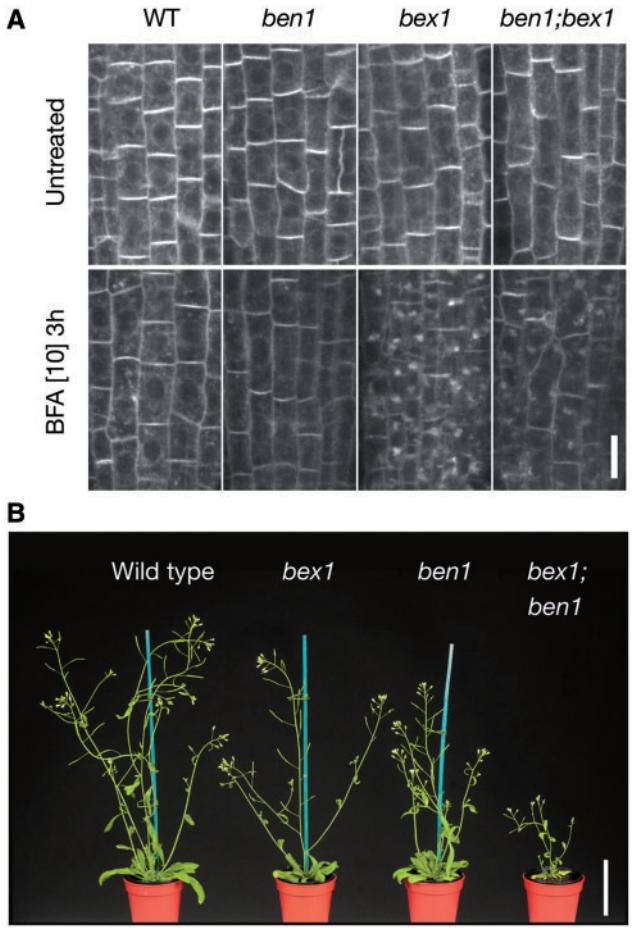

Fig. 5 The *ben1* mutation is epistatic to *bex1* in terms of endocytic recycling of PIN1–GFP. (A) PIN1–GFP signals in untreated (upper panels) and BFA-treated (10 μM, 3 h, lower panels) wild-type, single mutants and *ben1-1; bex1* double mutant seedlings. Scale bar = 10 μm. (B) Gross morphology of 38-day-old plants. Scale bar = 5 cm.

accumulated in agglomerated endosomes, which were reminiscent of the BFA compartments (**Supplementary Fig. S4B**). In contrast, when wild-type ARF1A1C or GDP-locked versions of ARF1A1C (T31N) fused to fluorescent proteins were expressed, no drastic change was observed in PIN1 localization (**Supplementary Fig. S4B**). These results suggest that expression of the ARF1^{Q71L} mutation has a strong inhibitory effect on PIN1 exocytosis. As expression of the mutated ARF1 also inhibits PIN2 trafficking (Xu and Scheres 2005), whose polar localization is essential for root gravitropism (Wiśniewska et al. 2006), we tested if the ARF1 mutants caused agravitropic root phenotypes. Overexpression of the ARF1 wild-type form did not interfere with root bending, whereas expression of ARF1 mutants with either a Q71L or T31N mutation reduced root bending (**Supplementary Fig. S5A**, **B**). Notably, this effect was not due to arrested root growth (**Supplementary Fig. S5C**), suggesting that ARF1 mutations abolished a trafficking process(es), which is physiologically relevant for asymmetric root growth.

To test further the developmental impact of the mutated ARF1A1C, the ARF1A1C^{Q71L} mutant was expressed under the control of a strong promoter from the *RPS5a* gene (Weijers et al. 2003). When the wild-type form of ARF1A1C driven by the *RPS5a* promoter was expressed as a control, the morphology of transgenic embryos was not affected (**Fig. 7A**). In contrast, when ARF1A1C^{Q71L} was expressed, the cell arrangement of the hypophysis (the basal part of embryos forming the embryonic root) was disorganized and cotyledon primordia did not develop (**Fig. 7B**).

To gain further insights into the molecular basis of those developmental defects, we localized PIN1 in the malformed embryos. In control specimens, the typical polar localization of PIN1 protein was detected at the 16-cell EP (embryo proper) stage and subsequently (**Fig. 7C**). When mutated ARF1A1C^{Q71L} was expressed, intracellular aberrant PIN1 signals were pronounced from the early stage onwards, with gradual loss of PIN1 basal localization in provascular cells and disorganization of embryonic patterning (**Fig. 7D**).

Consistent with PIN1 localization defects causing reduced directional auxin transport, the typical asymmetric auxin accumulation patterns as visualized by the *DR5rev::GFP* auxin response reporter (Friml et al. 2003b) were disrupted in ARF1A1C^{Q71L} embryos (**Fig. 7F**). Expression of another auxin-dependent developmental marker, *WOX5::GFP* (Blilou et al. 2005, Sarkar et al. 2007, Ding and Friml 2010), which in the wild-type situation is typically confined to lens-shaped QC cells (**Fig. 7G**), was dispersed in ARF1A1C^{Q71L} embryos (**Fig. 7H**). Taken together, our data indicate that disruption of ARF1 function has a strong impact on PIN1 trafficking, asymmetric auxin distribution and plant development already at its early, embryonic stages.

Discussion

To identify new molecular component(s) involved in exocytic trafficking of PIN1 protein, we have performed a fluorescence imaging-based screen to find Arabidopsis mutants which over-accumulate PIN1–GFP in BFA compartments. Among them we have identified *bex1*, harboring a dominant mutation in ARF1, which had a strong impact on PIN1 PM localization. By similar genetic approaches, endocytic trafficking-defective *ben* mutants as well as an exocytic trafficking-defective *bex5* mutant have been isolated (Tanaka et al. 2009, Feraru et al. 2012). These genetic screens so far identified a TGN/EE-localized ARF GEF BEN1/MIN7, Sec1-Munc18 (SM) protein BEN2/VPS45 and BEX5/RabA1b involved in endocytic recycling of PIN proteins (Tanaka et al. 2009, Feraru et al. 2012, Tanaka et al. 2013). Whereas *ben* mutants tend to accumulate less PIN proteins in intracellular compartments upon BFA treatment, *bex1* (this study) and *bex5* (Feraru et al. 2012) mutations cause over-accumulation of those endocytic cargos in BFA compartments. Both genetic screening approaches involved BFA treatments of different durations to detect distinct trafficking phenotypes.

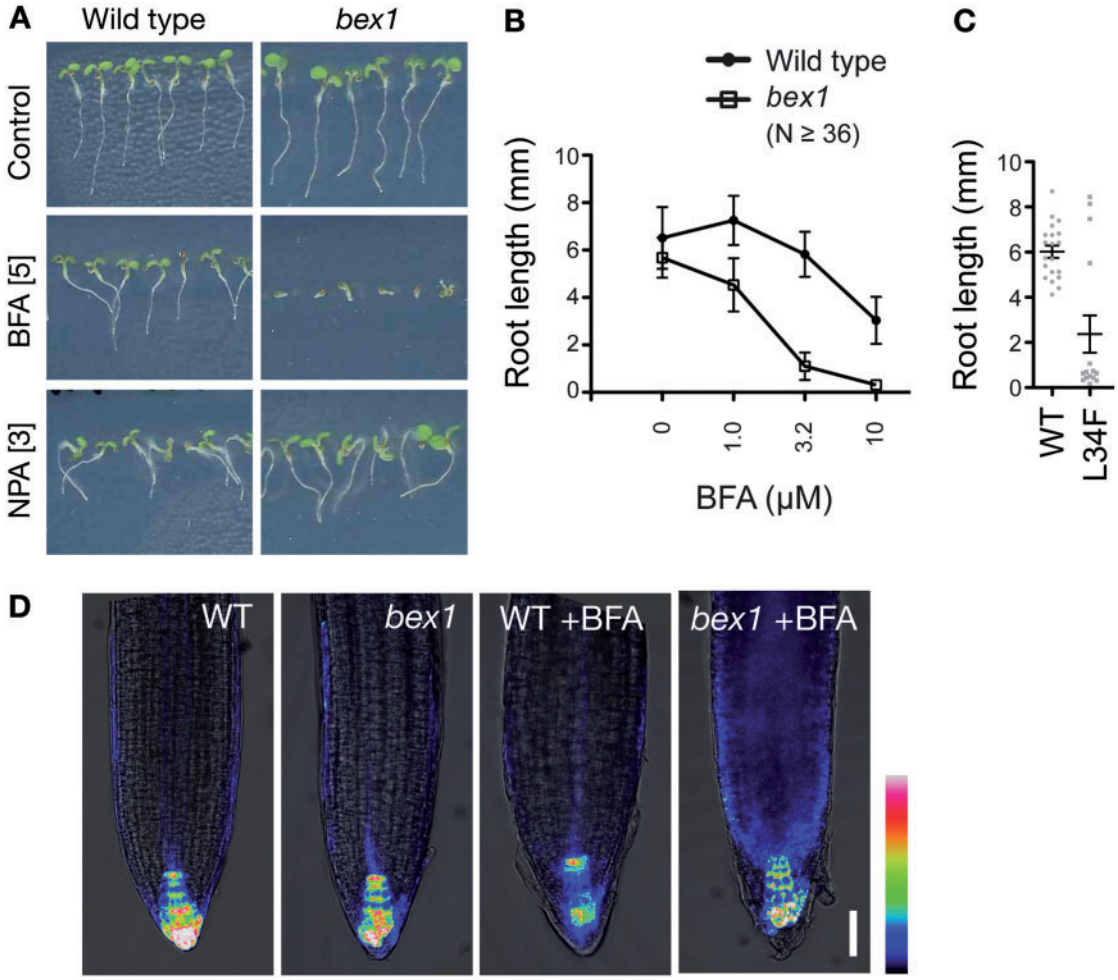

Fig. 6 Functional interference with ARF1A1C affects plant growth and development. (A) Gross morphology of seedlings grown on vertical solid plates with BFA (5 μM) and NPA (3 μM). Note that the *bex1* mutant does not grow in the presence of a low concentration of BFA. (B) Root length of wild-type and *bex1* seedlings grown for 5 d in the presence of different concentrations of BFA. (C) Root length of wild-type and T_2 seedlings of the *ARF1A1C*^{L34F}*-GFP* transgenic line grown vertically on BFA-containing solid plates (5 μM). Note that the BFA-hypersensitive phenotype of *bex1* was recapitulated by the *pARF1A1C::ARF1A1C*^{L34F}*-GFP* transgene. (D) Pattern of auxin responses as visualized by *DR5rev::GFP* expression in wild-type and *bex1* mutant seedlings grown on control media (left-hand panels) and BFA-containing media (25 μM, 20 h). The signal intensity of GFP is shown as color coding. Scale bar = 50 μm.

Thus, our results demonstrate that a fluorescence imaging-based screen involving the chemical treatment represents a manipulatable approach to identify molecular components involved in distinct trafficking processes.

By molecular mapping of the *bex1* mutation, we identified ARF1A1C as a critical component involved in PIN trafficking. Involvement of ARF components in PIN1 trafficking has been implicated from several lines of circumstantial evidence, including the strong inhibitory effect of BFA and mutations in potential regulators of ARF1 on PIN1 localization (Geldner et al. 2003, Scarpella et al. 2006, Richter et al. 2007, Teh and Moore 2007, Tanaka et al. 2009, Naramoto et al. 2009). Our results clearly demonstrated intracellular agglomeration of PIN1 proteins together with their depletion from the PM upon interference with ARF1A1C function (**Figs. 1**, **7**), suggesting the critical role of ARF1 in exocytosis/recycling of PIN1 to the PM.

However, PIN2 trafficking seems to be more complicated, as BFA treatment of *bex1* mutants revealed that not only recycling from endosomes to the PM, but also trafficking to the vacuole was severely inhibited. A proper root gravitropic response depends on the correct PIN2 polarity (Wiśniewska et al. 2006). Multiple trafficking processes, such as regulated degradation (Abas et al. 2006) including ubiquitination-dependent trafficking to the vacuole (Leitner et al. 2012) as well as localized endocytosis, polar exocytosis and limited lateral diffusion at the PM, contribute to the correct polar PIN2 localization in the root epidermis (Kleine-Vehn et al. 2011). Therefore, as inhibition of ARF1 function resulted in root gravitropism defects (**Supplementary Fig. S5**), it remains to be determined which of the trafficking processes disrupted by the ARF1 mutation contributed most to agravitropic root growth.

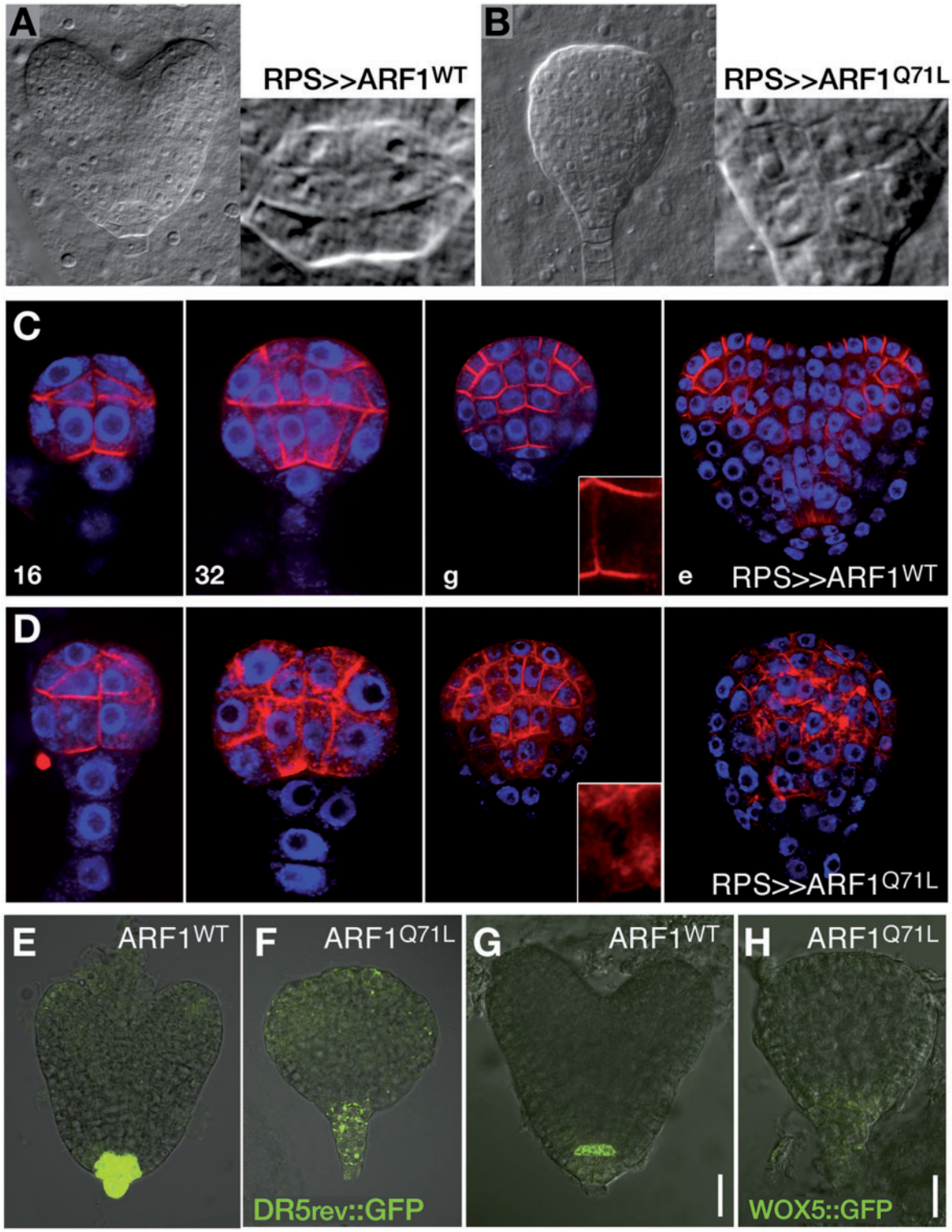

Fig. 7 ARF1 mutation causes intracellular agglomeration of PIN1 and modifies auxin-dependent gene expression. (A, B) Effects of ARF1 expression driven by the *RPS5a::Gal4-VP16* activator line (ACT RPS5A) genetically crossed with *UAS::ARF1*^{WT} (A) and *UAS::ARF1*^{Q71L} (B). Expression of the GTP-locked version of ARF1A1C (Q71L) causes severe embryonic patterning defects. Magnified views indicate the basal parts of embryos. (C, D) PIN1 immunolocalization (Cy3, red) in developing embryos at various stages. Nuclei are visualized with a counterstain [4′,6-diamidino-2-phenylindole (DAPI), blue]. Abbreviations are: 16, 16-cell EP stage; 32, 32-cell EP stage; g, globular stage; e, early heart stage. (E, F) Auxin distribution maxima as visualized by the *DR5rev::GFP* reporter in RPS5>>ARF1^{WT} (E) and RPS5>>ARF1^{Q71L} (F) embryos. (G, H) Expression pattern of *WOX5::GFP* in RPS5>>ARF1^{WT} (G) and RPS5>>ARF1^{Q71L} (H) embryos. Scale bars = 20 μm.

As expression of mutated ARF1 proteins impacts strongly on intracellular trafficking as well as plant growth and development, regulation of ARF1 activity is of key importance. Performing its role in the subcellular machinery, ARF1 oscillates between GDP- and GTP-bound states, and this cycling is essential in regulating ARF1 function by changing the membrane binding state and effector binding (D'Souza-Schorey and Chavrier 2006). Consistently, ARF1A1C^{Q71L}–YFP localizes to endosomes, whereas ARF1A1C^{T31N}–cyan fluorescent protein (CFP) exhibits cytosolic localization and behaves as a dominant negative mutant (Xu and Scheres 2005) (**Supplementary Fig. S4**). Interestingly, overexpression of ARF1A1C^{L34F}–GFP caused intracellular agglomeration of its own GFP signals in the absence of BFA (**Fig. 4**; **Supplementary Fig. S3**). In this respect, L34F mutation seems to have promoted the ARF1 to the membrane binding-competent state, reminiscent of Q71L mutation rather than T31N mutation. Consistent with previous reports (Stefano et al. 2006, Matheson et al. 2007), we observed depletion of SEC21/γ-COP following induction of ARF1^{T31N} mutant expression, whereas expression of the ARF1^{Q71L} mutant did not decrease SEC21/γ-COP signals. We also observed differential effects of ARF1^{Q71L} and ARF1^{T31N} in terms of PIN1 agglomeration. However, due to technical limitations of the overexpression experiment, we cannot rule out the possibility that the absence of visible effects might be due to insufficient expression of the mutated proteins. The impact of the L34F mutation on ARF1 structural change also remains to be determined.

GDP to GTP exchange of ARF-related GTPases is generally catalyzed by ARF GEFs, most of which share a conserved Sec7 domain (Gillingham and Munro 2007). It has been proposed that GTP-bound ARF1 and an as yet unidentified component recruit ARF GEF to the membrane, thereby promoting further recruitment of ARF1-GDP for GDP–GTP exchange in yeast (Richardson and Fromme 2012). The Arabidopsis genome contains eight genes encoding Sec7 domain proteins (Anders and Jürgens 2008). ARF GEF GNOM of Arabidopsis has activity to exchange GDP and GTP on mammalian ARF1 protein (Steinmann et al. 1999). So far, the roles of several ARF GEFs, including GNOM, GNL1 and BEN1/MIN7, in secretion, endocytosis, endosomal trafficking and/or recycling have been demonstrated (Geldner et al. 2003, Richter et al. 2007, Teh and Moore 2007, Tanaka et al. 2009, Naramoto et al. 2010). In this respect, it is interesting that ARF1A1C partially co-localized with the functionally characterized ARF GEFs GNOM, GNL1 and BEN1/MIN7. We speculate that ARF1A1C might be regulated in distinct compartments by different ARF GEFs, as has been likewise suggested in yeast (Richardson and Fromme 2012). If this is the case, it is expected that mutation of *bex1* might enhance the phenotypes caused by ARF GEF interference. Indeed, in terms of plant growth and development, the *bex1* mutant grown on BFA-containing media had strong growth retardation and the *ben1*; *bex1* double mutant exhibited severe dwarfism (**Figs. 5**, **6**). It is also worth noting that strong inhibition of ARF1 function during embryogenesis resulted in pronounced intracellular accumulation and diminished PM signals of PIN1 in provascular cells. In terms of PIN1 PM distribution, this phenotype was distinct from that of ARF GEF single mutants reported so far. Taken together, these results support the roles of ARF1A1C in multiple trafficking processes involving different ARF GEFs.

In summary, we have demonstrated that interference with the functions of ARF1A1C had a strong impact on PIN1 PM distribution, vacuolar targeting of PIN2–GFP and consequently on multiple auxin-dependent developmental processes including embryogenesis and root gravitropic responses. Being localized in different intracellular compartments labeled by different ARF GEFs, it is likely that ARF1A1C is regulated by multiple ARF GEFs to regulate specific trafficking processes. Because of its evolutionary conservation and strong impact on membrane trafficking, the roles of ARF1 in plant cells have been extensively studied. Inhibition of ARF1 function results in various trafficking defects including intracellular accumulation of PM cargos (Lee et al. 2002), mislocalization of Golgi-localized proteins to the ER (Takeuchi et al. 2002, Xu and Scheres 2005), endocytosis defects and mistargeting of ROP GTPase (Xu and Scheres 2005). However, ARF1 has not been identified as a regulator of PIN1 exocytosis, prior to this study. AP1 is one of the ARF1 effectors functioning as a coat component, regulating vesicle budding at the TGN in animals. Recently, roles of AP1 in post-Golgi trafficking have also been demonstrated in Arabidopsis (Park et al. 2013, Teh et al. 2013, Wang et al. 2013). In addition, several other molecular players involved in PIN1 exocytosis, including exocyst (Drdova et al. 2013), ICR1/RIP1 (Hazak et al. 2010) and BEX5/RabA1b (Feraru et al. 2012), have been identified. It remains to be determined whether these components and ARF1A1C function in the same trafficking pathway. Further identification of ARF1A1C effectors would also shed light on the downstream events of PIN exocytosis, including vesicle budding, transport, tethering and fusion at the target membrane.

Materials and Methods

Plant materials, genetic screening and phenotypic analysis

The following *Arabidopsis thaliana* mutants and transgenic lines have been described previously: *PIN1-GFP* (Benková et al. 2003), *ben1-1*; *PIN1-GFP* (Tanaka et al. 2009), *DR5rev::GFP* (Friml et al. 2003b), *PIN2-GFP*; *eir1* (Xu and Scheres 2005), *pARF1::ARF1-GFP* (Xu and Scheres 2005), *UAS::ARF1^{WT} UAS::ARF1^{Q71L}* (Xu and Scheres 2005), *HSP::ARF1^{WT}-GFP*, *HSP::ARF1^{Q71L}-YFP*, *HSP::ARF1^{T31N}-CFP* (Xu and Scheres 2005), *WOX5::GFP* (Blilou et al. 2005), ACT RPS5A (Weijers et al. 2003), *GNOM-GFP* (Geldner et al. 2003) and *GNL1-YFP* (Richter et al. 2007, Teh and Moore 2007). Plants were grown as described previously (Tanaka et al. 2009). For genetic screening, M_2 seedlings, harboring *PIN1-GFP* descending from approximately 1,200 M_1 plants, were treated with 50–100 μM BFA for 16–20 h.

Approximately 39,200 M_2 seedlings were inspected by epifluorescence microscopy. *PIN2-GFP; eir1* was introduced into the *bex1* mutant by genetic crossing. *DR5rev::GFP* and *WOX5::GFP* were introduced into the ACT RPS5A line by genetic crossing. Measurements of root length and clearing of embryos were performed as described previously (Tanaka et al. 2007, Tanaka et al. 2009).

Chemical treatment, immunodetection and microscopy

Treatment with BFA (Invitrogen B7450) was performed as described previously (Tanaka et al. 2009). Whole-mount immunolocalization on Arabidopsis roots was performed as described previously (Friml et al. 2003a). Antibodies were diluted as follows: rabbit anti-PIN1 (1 : 1,000) (Paciorek et al. 2005), goat anti-PIN1 (1 : 400; Santa Cruz sc-27163), rabbit anti-PIN2 (1 : 1,000) (Abas et al. 2006), rabbit anti-ARF1A1C (1: 1,000; agrisera AS08325), rabbit anti-SEC21 (1 : 1,000, agrisera AS08327), rabbit anti-BiP2 (1 : 1,000, agrisera AS09481), mouse anti-GFP (1 : 1,000; Roche 11 814 460 001), Alexa 488-conjugated secondary anti-mouse (1116 : 6 : 6 : 00; Invitrogen A11029), Cy3-conjugated secondary anti-rabbit (1 : 600; Sigma C2306) and DyLight 649-conjugated secondary anti-goat (1 : 400; Jackson Immuno Research 705-495-147) antibodies. For detection of GFP in the vacuole, seedlings were kept in darkness for 5 h to suppress degradation of GFP (Tamura et al. 2003, Kleine-Vehn et al. 2008b). Fluorescence signals were imaged using Olympus FV1000, Carl Zeiss LSM5 exciter or LSM710 confocal microscopes. For quantification of intracellular PIN1–GFP accumulation, the number of agglomerated PIN1–GFP signals (cross-sectional area $>1\,\mu m^2$) in each root vascular cell was scored using imageJ software (National Institute of Health, USA). At least 87 cells from each treatment were analyzed. For co-localization studies, confocal images of root epidermal cells were acquired with a $\times 63$ oil immersion objective lens (NA 1.4). Images from at least three roots for each combination were subjected to analysis. The co-localization coefficient was calculated with ZEN 2009 software (Carl Zeiss). Immunoblot analysis was performed essentially as described (Sasabe et al. 2011). Protein extracts were prepared from whole wild-type and transgenic plants (13 d old) with modified TG150 buffer (prepared with 0.5% Triton X-100 and 0.1% Tween-20 instead of 0.1% Triton X-100) and equal amounts of extracts (20 μg) were separated by SDS–PAGE (11% acrylamide). Antibodies were diluted as follows: rabbit anti-ARF1A1C (1 : 1,000; agrisera AS08325), mouse anti-GFP antibody (1 : 1,000; Roche 11 814 460 001), anti-rabbit horseradish peroxidase (HRP; 1 : 5,000; GE NA 934VS) and anti-mouse HRP (1 : 2,000; GE NA 931VS).

Molecular cloning of the *BEX1* gene and DNA construction

Polymorphic F_2 seedlings were obtained from crossing the *bex1* mutant with a Landsberg *erecta* ecotype. For mapping, we selected *bex1* homozygous and segregating wild-type plants based on the phenotypes at the F_2 and/or F_3 generations by inspecting BFA-hypersensitive PIN1–GFP localization or growth defects. DNA was isolated from a total of 399 F_2 plants or their progeny and subjected to polymorphism analysis. The *bex1* mutation was mapped between two markers, F14M4-MunI (19.348 Mb) and T8I13-MboII (19.390 Mb), on chromosome 2. To detect the *bex1* mutation by PCR genotyping, the relevant region was amplified by mutagenic PCR using primers At2g47170-71F-XmnI, 5′-GTCTCGATGCTGCTGGTAAGACGAATAT-3′; and At2g47170-211R, 5′-CAACAGTAAATCACATCCATCATACA-3′, and digested with *Xmn*I restriction enzyme. To generate the *pARF1A1C::ARF1A1C^{L34F}-GFP* construct, the *ARF1A1C* gene including 2.07 kb of upstream sequence was amplified from *bex1* genomic DNA with the following primers: ARF1A1C-pro2071GWB1F, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAGTAACAAGACGGATCCTT-3′; and ARF1A1C-C-fusion-GWB2R, 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTTGCCTGCGAAACATACATTCA-3′. The PCR product was cloned into a binary vector pK7FWg.0 (https://gateway.psb.ugent.be/) (Karimi et al. 2002) by using Gateway technology (Invitrogen). The construct was transformed into Col-0 wild-type plants, and transgenic plants were selected on solid media containing kanamycin (25 mg l^{-1}). To generate *P35S::BEN1-GFP* transgenic plants, the *P35S::BEN1-GFP* region from pEPA-cBEN1-GFP (Tanaka et al. 2009) was cloned into the binary vector pMLBART (Eshed et al. 2001) to generate pMLBART-cBEN1-GFP. The construct was transformed into *ben1-2* plants (Tanaka et al. 2009). Transgenic plants were selected on solid medium containing phosphinothricin (15 mg l^{-1}).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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